Possible Neurotoxicity of Titanium Dioxide Nanoparticles in a Subacute Rat Model

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Abstract
Titanium dioxide nanoparticles (TiO_2 NPs) have many industrial applications and also appear in various consumers' goods including foods and medicines. This widespread application raises the question of a potential occupational, environmental and/or intentional human exposure and health hazard. Motility of NPs within the organism and surface reactivity of TiO_2 NPs suggests, among others, potential nervous system tixicity. In the present work, rats were intratracheally exposed to TiO_2 NPs and functional changes in the nervous system were examined using electrophysiological, behavioural and biochemical methods. The results verified to some extent the neurotoxicity of nano-titanium but also underlined the need for further investigations.

Introduction
Thanks to the recent advances in nanosciences and nanotechnology, more and more industrial processes and products involve the presence of NPs, that is particles with <100 nm typical diameter. Beside research applications NPs have had their entry in the fields of health care, energy production, agriculture and environmental protection [1] and are being released in the environment [2]. The physical, chemical and biological properties of nanoparticulate substances differ from those seen in other physical states, leading to biological, and hence, toxicological, interactions not seen with more conventional materials, which also means novel health risks [3].
Particles of TiO_2 – in the micrometer, and newly, nanometer, range – have been used in paints, various coatings, plastics, food (E171), toothpastes, skin care products and in sunscreens as radiation blocking agent [4]. The anatase form of TiO_2 has photocatalytic properties in UV light, it is applied as a sterilizer and deodorant, and as additive in paints and building materials to reduce the level of air pollutants [5].
This broad and growing range of application raises questions about possible health risks involved. Today, chemical safety is a primary requirement so toxicological evaluation of novel materials should precede their application or at least go in parallel [4, 5]. Contradictory findings are found in the literature on the absorption and migration, including penetration to the brain, of TiO_2 NPs after pulmonary, dermal, oral etc. exposure. That nervous system effects can be expected, is indicated by the oxidative stress generating potency of TiO_2 NPs, and by biochemical, histological and functional alterations observed mostly in mice [4].
In the present work, rats were exposed to nano-TiO_2 by the intratracheal route, and functional alterations of the nervous system were detected by electrophysiological and behavioral methods. The aims were determining the suitable dose range and testing the applicability of the methodological approach proven in toxicological work with other metal oxide NPs [6].

Experimental

Young adult SPF Wistar rats were used, obtained from Toxi-Coop Ltd. (Hungary). The animals (with 170±20g body weight at start) were kept in polypropylene cages (3-4 rats/cage) under GLP-equivalent conditions. The rats had free access to fresh water throughout the experiment. Based on previous data, the daily food ration (rat chow) was 30g/animal. (Ssniff R/M-Z+H rat chow, Toxi-Coop Ltd., Hungary). The animals' body weight was measured daily.

The rats were randomly distributed to 5 groups of 10 rats each based on their performance in preliminary spontaneous exploratory activity test. Rats in the control group (C) were totally untreated while vehicle control rats (VC) received a 1% solution of HEC (hydroxyethyl cellulose) in phosphate-buffered saline by intratracheal instillation (see [6]). Treated rats received TiO_2 nanoparticles (NPs) suspended in the HEC-containing vehicle and instilled, the doses were 1 mg/kg body weight (low dose, group L), 3 mg/kg (medium dose, M), and 10mg/kg (high dose, H). The TiO_2 NPs were spherical with <50 nm diameter. Treatment was done every day during a 28 days period, between 8:00 and 10:00 a.m.

The rats' spontaneous exploratory activity was tested, at start and after the last treatment day, in an open field (OF) box (Conducta 1.0 System, Experimetria Ltd., Hungary). The animals were, one by one, placed into the centre of the box, and 16 motility parameters – ambulation distance, time and count; local activity time and count; vertical activity (rearing) time and count; immobility time and count – were measured in one 10 min session.

On the day following the final OF session, the rats were prepared for electrophysiological recording in urethane anesthesia (1000 mg/kg b.w. ip). The skull was opened over the left hemisphere, and silver electrodes were placed on the the primary somatosensory (SS) visual (VIS) and auditory (AUD) areas. Spontaneous electrical activity was recorded from these sites simultaneously for 6 min, and the relative spectral power of the frequency bands was determined. Then, sensory stimuli were applied to obtain sensory evoked potentials (EPs, for details see [6]) Fifty stimuli of each modality per rat were applied. For VIS and AUD stimulation, 1 Hz frequency was applied, and for SS stimulation 1, 2 and 10 Hz. The 50 EPs were averaged and onset latency was measured. To see changes in peripheral nerves, the tail nerve was electrically stimulated at the tail base (3-4 V; 0.05 ms) and the nerve action potentials were recorded 50 mm more distally. Conduction velocity was calculated from the response latency, and refractory period, from the extra latency of the second action potential obtained by double stimulation. The complete recording and evaluation was performed by the software NEUROSYS 1.11 (Experimetria Ltd., Hungary).

After that, the rats were sacrificed by an overdose of urethane, were dissected and organs were weighed. Relative organ weights were calculated to 1/100 body weight. From each group, samples of 3 randomly chosen rats were kept for metal level determination, and of another 3, for biochemical measurements.

From the functionaal, biochemical and general toxicological data, group means were calculated and checked for normality by the Kolmogorov-Smirnov test. The main statistical test used was parametric one-way ANOVA or non-parametric Kruskal-Wallis method. Post hoc analysis of group differences was done by Tukey test and the paired Mann-Whitney U test with Holm correction. SPSS 17.0 (IBM Corporation, U.S.A.) was used.

Results and Discussion

The general toxicity on TiO_2 NP exposure was detected by means of changes in body weight gain and organ weights. The effect of treatment was seen, both in the vehicle control group (VC) and the TiO_2 NP-treated groups, from the 2^{nd} week on. By the end of the treatment

period, the total weight gain over the 6 weeks was significantly lower in the groups receiving the two higher doses of nano-TiO_2 (Table 1).
The relative weight of the lungs increased significantly vs. C and VC in the groups M and L. Relative brain weight decreased significantly in groups M and H vs. C. For both organs, the maximal change was seen in group M.

Table 1. Body weight gain (g) of the control and nano-TiO_2 treated rats over the 6 weeks treatment. Mean±SD, n=10. *: p<0.05 vs. C.

Groups	**C**	**VC**	**L**	**M**	**H**
Body weight gain	213.6±34.9	177.4±43.4	187.7±37.2	142.2±16.5*	165.2±26.6*

Out of the electrophysiological parameters investigated, the band spectrum of the ECoG was shifted slightly to higher frequencies in the nano-TiO_2 treated groups but this was below significance. The changes in EP latency were, however, significant. As seen in Fig. 1, latency increased also in VC vs. C but the changes in the treated groups were mostly significant vs., both controls. It is also conspicuous that, similar to body weight gain, the biggest change of SS and VIS EP latency was seen with the medium dose (group M) not the high dose (H). This was somewhat in parallel with the Ti levels of the tissue samples (Table 2).
In the tail nerve, the conduction velocity was significantly reduced but the length of relative refractory period changed less markedly (Fig. 2.)

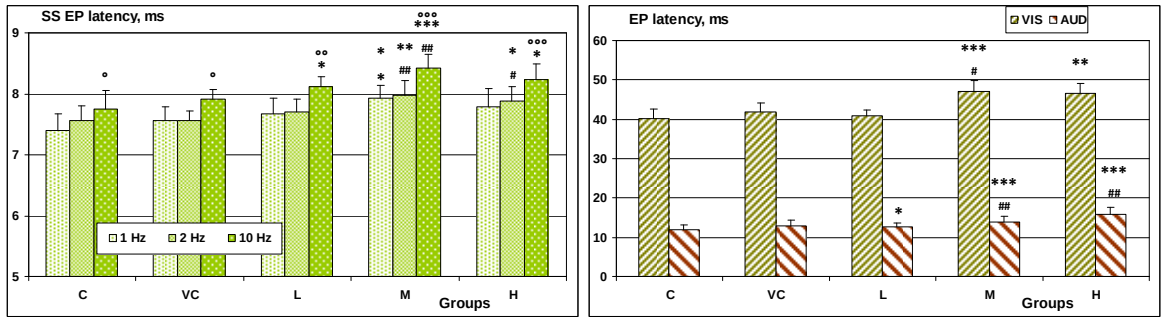

Figure 1. Latency of the somatosensory EP (at various frequencies, left) and the visual and auditory EP (right) after 6 weeks nano-TiO_2 treatment. Mean+SD, n=10. *, **, ***: p<0.05, 0.01, 0.001 vs. C.; $^{\#}$, $^{\#\#}$: p<0.05, 0.01 vs. VC; °, °°, °°°: p<0.05, 0.01, 0.001 vs. 1 Hz stimulation within the same group.

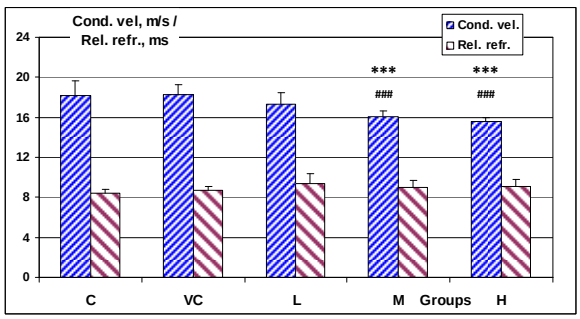

Figure 2. Conduction velocity and relative refractory period of the tail nerve after 6 weeks nano-TiO_2 treatment. Mean+SD, n=10. ***: p<0.001 vs. C.; $^{\#\#\#}$: p< 0.001 vs. VC

In the OF activity, the treated rats tended to spend more time in the corner zones of the field than those without nano-TiO_2 exposure although the difference between group C and VC was not negligible. Activity in the first minute, showing immediate reaction of the rat on the novel environment, also showed the preference of treated rats to the corners, indicating increased level of anxiety (Fig. 3). The tissue Ti levels (Table 2) and the relative organ weights (not shown) indicated that most of the nano-TiO_2 remained in the lungs and that the amount reaching other organs had the same non-linear dose dependence seen also in several functional alterations, pointing to a causative role of the NPs.

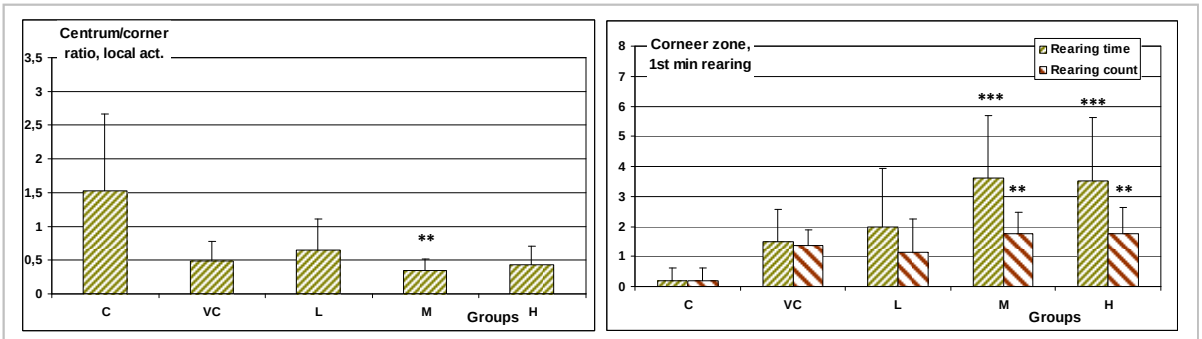

Figure 3. Open field activity of the control and treated rats: Ratio of the local activity count in the central and corner zones of the OF box over the 10 min sesssion; right: Activity in the corner zones in the 1st min of the session. Mean+SD, n=10. Significance marking as above.

Table 2. Ti levels (µg/kg wet tissue) of various organs (Mean±SD, n=3)

Groups	Lungs	Blood	Liver	Brain
VC	1.494±0.925	1.543±0.941	3.953±3.306	2.846±1.619
L	43.232±6.146	3.169±1.852	1.263±0.455	2.916±2.126
M	42.314±6.467	3.486±2.160	2.680±2.959	6.235±3.188
H	81.488±4.261	1.000±0.000	1.691±1.196	1.241±0.418

The intensity of thiobarbiturate reaction, as a measure of oxidative damage of lipids, was dependent on Ti dose in the lungs and liver, but not in the brain. This contradicts to the functional alterations and metal levels and argues more for an indirect mechanism of action.

Conclusion

It was possible to detect functional neurotoxicity of TiO_2 NP given to the rats. The results, however, raise questions to be answered in further experiments such as the dose dependence and the relationship of neuro-functional and biochemical changes to the external and internal nano-TiO_2 dose.

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